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OPIOID RECEPTOR GENES

This invention was made with Government support under Grant No. DA05010 awarded by the Alcohol, Drug Abuse and Mental Health Administration. The Government has certain rights in the invention.

Cross-Reference to Related Applications

This application is a continuation-in-part of U.S. Serial No. 08/387,707 filed 13 February 1995 (the National Phase application of PCT US 93/07665 filed 13 August 1993) which is a continuation-in-part of U.S. Serial No. 07/929,200 filed 13 August 1992. The contents of these applications are incorporated herein by reference.

Technical Field

The invention relates to substances involved in vertebrate nervous systems, and in particular to the opioid receptors and receptor-like proteins (also referred to as opioid receptors herein) and activities mediated thereby. Accordingly, the invention concerns recombinant materials useful for the production of opioid receptors, the receptor as a diagnostic tool, therapeutic and diagnostic compositions relevant to the receptor, and methods of using the receptor to screen for drugs that modulate the activity of the receptor.

Background Art

The term "opioid" generically refers to all drugs, natural and synthetic, that have morphine-like actions. Formerly, the term "opiate" was used to designate drugs derived from opium, e.g., morphine, codeine, and many semi-synthetic congeners of morphine. After the isolation of peptide compounds with morphine-like actions, the term opioid was introduced to refer generically to all drugs with morphine-like actions. Included among opioids are various peptides that exhibit morphine-like activity, such as endorphins, enkephalins and dynorphins. However, some sources have continued to use the term "opiate" in a generic sense, and in such contexts, opiate and opioid are interchangeable. Additionally, the term opioid has been used to refer to antagonists of morphine-like drugs as well as to characterize receptors or binding sites that combine with such agents.

Opioids are generally employed as analgesics, but they may have many other pharmacological effects as well. Morphine and related opioids produce their major effects on the central nervous and digestive systems. The effects are diverse, including analgesia, drowsiness, mood changes, respiratory depression, dizziness, mental clouding, dysphoria, pruritus, increased pressure in the biliary tract, decreased gastrointestinal motility, nausea, vomiting, and alterations of the endocrine and autonomic nervous systems.

A significant feature of the analgesia produced by opioids is that it occurs without loss of consciousness. When therapeutic doses of morphine are

given to patients with pain, they report that the pain is less intense, less discomforting, or entirely gone. In addition to experiencing relief of distress, some patients experience euphoria. However, when morphine in a selected pain-relieving dose is given to a pain-free individual, the experience is not always pleasant; nausea is common, and vomiting may also occur. Drowsiness, inability to concentrate, difficulty in mentation, apathy, lessened physical activity, reduced visual acuity, and lethargy may ensue.

The development of tolerance and physical dependence with repeated use is a characteristic feature of all opioid drugs, and the possibility of developing psychological dependence on the effect of these drugs is a major limitation for their clinical use. There is evidence that phosphorylation may be associated with tolerance in selected cell populations (Louie, A. et al. Biochem Biophys Res Comm (1988) 152:1369-75).

Acute opioid poisoning may result from clinical overdosage, accidental overdosage, or attempted suicide. In a clinical setting, the triad of coma, pinpoint pupils, and depressed respiration suggest opioid poisoning. Mixed poisonings including agents such as barbiturates or alcohol may also contribute to the clinical picture of acute opioid poisoning. In any scenario of opioid poisoning, treatment must be administered promptly.

The opioids interact with what appear to be several closely related receptors. Various inferences have been drawn from data that have attempted to correlate pharmacologic effects with the interactions of opioids with a particular constellation of opioid receptors

(Goodman and Gilman's, THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 7th ed, pp. 493-95 (MacMillan 1985)). For example, analgesia has been associated with mu and kappa receptors. Delta receptors are believed to be involved in alterations of affective behavior, based primarily on the localization of these receptors in limbic regions of the brain. Additionally, activation, *e.g.*, ligand binding with stimulation of further receptor-mediated responses, of delta opioid receptors is believed to inhibit the release of other neurotransmitters. The pathways containing relatively high populations of delta opioid receptor are similar to the pathways implicated to be involved in Huntington's disease. Accordingly, it is postulated that Huntington's disease may correlate with some effect on delta opioid receptors.

Two distinct classes of opioid molecules can bind opioid receptors: the opioid peptides (*e.g.*, the enkephalins, dynorphins, and endorphins) and the alkaloid opiates (*e.g.*, morphine, etorphine, diprenorphine and naloxone). Subsequent to the initial demonstration of opiate binding sites (Pert, C.B. and Snyder, S.H., Science (1973) 179:1011-1014), the differential pharmacological and physiological effects of both opioid peptide analogues and alkaloid opiates served to delineate multiple opioid receptors. Accordingly, three anatomically and pharmacologically distinct opioid receptor types have been described: delta, kappa and mu. Furthermore, each type is believed to have sub-types (Wollemann, M., J Neurochem (1990) 54:1095-1101; Lord, J.A., et al., Nature (1977) 267:495-499).

All three of these opioid receptor types appear to share the same functional mechanisms at a cellular level. For example, the opioid receptors cause inhibition of adenylate cyclase, and inhibition of neurotransmitter release via both potassium channel activation and inhibition of Ca^{2+} channels (Evans, C.J., In: Biological Basis of Substance Abuse, S.G. Korenman & J.D. Barchas, eds., Oxford University Press (in press); North, A.R., et al., Proc Natl Acad Sci USA (1990) 87:7025-29; Gross, R.A., et al., Proc Natl Acad Sci USA (1990) 87:7025-29; Sharma, S.K., et al., Proc Natl Acad Sci USA (1975) 72:3092-96). Although the functional mechanisms are the same, the behavioral manifestations of receptor-selective drugs differ greatly (Gilbert, P.E. & Martin, W.R., J Pharmacol Exp Ther (1976) 198:66-82). Such differences may be attributable in part to the anatomical location of the different receptors.

Delta receptors have a more discrete distribution within the mammalian CNS than either mu or kappa receptors, with high concentrations in the amygdaloid complex, striatum, substantia nigra, olfactory bulb, olfactory tubercles, hippocampal formation, and the cerebral cortex (Mansour, A., et al., Trends in Neurosci (1988) 11:308-14). The rat cerebellum is remarkably devoid of opioid receptors including delta opioid receptors.

Several opioid molecules are known to selectively or preferentially bind delta receptors. Of the vertebrate endogenous opioids, the enkephalins, particularly met-enkephalin and leu-enkephalin, appear to possess the highest affinity for delta receptors, although

the enkephalins also have high affinity for mu receptors. Additionally, the deltorphans, peptides isolated from frog skin, comprise a family of opioid peptides that have high affinity and selectivity for delta receptors (Erspamer, V., et al., Proc Natl Acad Sci USA (1989) 86:5188-92).

A number of synthetic enkephalin analogues are also delta receptor-selective including ($D\text{-}Ser^2$) leucine enkephalin Thr (DSLET) (Garcel, G. et al. FEBS Lett (1980) 118:245-247) and ($D\text{-}Pen^2$, $D\text{-}Pen^5$) enkephalin (DPDPE) (Akiyama, K. et al., Proc Natl Acad Sci USA (1985) 82:2543-2547).

Recently a number of other selective delta receptor ligands have been synthesized, and their bioactivities and binding characteristics suggest the existence of more than one delta receptor subtype (Takemori, A.E., et al., Ann Rev Pharm Toxicol, (1992) 32:239-69; Negri, L., et al., Eur J Pharmacol (1991) 196:355-335; Sofuoglu, M., et al., Pharmacologist (1990) 32:151).

Although the synthetic pentapeptide 2dAla, 5dLeu enkephalin (DADLE) was considered to be delta-selective, it also binds equally well to mu receptors. The synthetic peptide $D\text{-}Ala^2\text{-}N\text{-}Me\text{-}Phe^4\text{-}Gly\text{-}ol^5$-enkephalin (DAGO) has been found to be a selective ligand for mu-receptors.

The existence of multiple delta opioid receptors has been implied not only from the pharmacological studies addressed above, but also from molecular weight estimates obtained by use of irreversible affinity ligands. Molecular weights for the delta opioid receptor that range from 30 kDa to 60 kda (Evans, C.J., *supra*; Evans, C.J. *et al.*, Science (1992) 258:1952-1955, which document

corresponds to the disclosure of the priority document of the present application; Bochet, P. et al., Mol Pharmacol (1988) 34:436-43). The various receptor sizes may represent alternative splice products, although this has not been established.

Many studies of the delta opioid receptor have been performed with the neuroblastoma/glioma cell line NG108-15, which was generated by fusion of the rat glial cell line (C6BU-1) and the mouse neuroblastoma cell line (N18-TG2) (Klee, W.A. and Nirenberg, M. A., Proc Natl Acad Sci USA (1974) 71:3474-3477). The rat glial cell line expresses essentially no delta opioid receptors, whereas the mouse neuroblastoma cell line expresses low amounts of the receptor. Thus, it has been suggested that the delta receptor in the NG108-15 cells is of mouse chromosomal origin (Law, Mol Pharm (1982) 21:438-91). Each NG108-15 cell is estimated to express approximately 300,000 delta-receptors. Only delta-type opioid receptors are expressed, although it is not known whether these represent more than a single subtype. Thus, the NG108-15 cell line has served to provide considerable insight into the binding characterization of opioid receptors, particularly delta opioid receptors. However, the NG108-15 cell line is a cancer-hybrid and may not be completely representative of the delta receptor in endogenous neurons due to the unique cellular environment in the hybrid cells.

An extensive literature has argued that the opioid receptors are coupled to G-proteins (see, *e.g.*, Schofield, P.R., et al., EMBO J (1989) 8:489-95), and are thus members of the family of G-protein coupled receptors.

G-proteins are guanine nucleotide binding proteins that couple the extracellular signals received by cell surface receptors to various intracellular second messenger systems. Identified members of the G-protein-coupled family share a number of structural features, the most highly conserved being seven apparent membrane-spanning regions, which are highly homologous among the members of this family (Strosberg, A.D., Eur J Biochem (1991) 196:1-10). Evidence that the opioid receptors are members of this family includes the stimulation of GTPase activity by opioids, the observation that GTP analogues dramatically effect opioid and opiate agonist binding, and the observation that pertussis toxin (which by ADP ribosylation selectively inactivates both the Gi and Go subfamilies of G-proteins) blocks opioid receptor coupling to adenylate cyclase and to K^+ and Ca^{2+} channels (Evans, C.J., *supra*).

The members of the G-protein-coupled receptor family exhibit a range of characteristics. Many of the G-protein-coupled receptors, *e.g.*, the somatostatin receptor and the angiotensin receptor, have a single exon that encodes the entire protein coding region (Strosberg *supra*; Langord, K., et al., Biochem Biophys Res Comm (1992) 138:1025-1032). However, other receptors, such as substance P receptor and the dopamine D-2 receptor, contain the protein coding region. The D-2 receptor is particularly interesting in that alternate splicing of the gene gives rise to different transcribed products (*i.e.*, receptors) (Evans, C.J., *supra*; Strosberg, *supra*). Interestingly, somatostatin ligands are reported to bind to opioid receptors (Terenius, L., Eur J Pharmacol (1976)

38:211; Mulder, A.H., et al., Eur J Pharmacol (1991) 205:1-6) and, furthermore, to have similar molecular mechanisms (Tsunoo, A., et al., Proc Natl Acad Sci USA (1986) 83:9832-9836).

In previous efforts to describe and purify opioid receptors, two clones have been described that were hypothesized either to encode a portion of or entire opioid receptors. The first clone, which encodes the opiate binding protein OBCAM (Schofield *et al.*, *supra*), was obtained by utilizing a probe designed from an amino acid sequence of a protein purified on a morphine affinity column. OBCAM lacks any membrane spanning domains but does have a C-terminal domain that is characteristic of attachment of the protein to the membrane by a phosphatidylinositol (PI) linkage. This feature, which is shared by members of the immunoglobulin superfamily, is not common to the family of receptors coupled to G-proteins. Thus, it has been proposed that OBCAM is part of a receptor complex along with other components that are coupled to G-proteins (Schofield *et al.*, *supra*). At present, however, there is no direct evidence for such a complex.

A second proposed opioid receptor clone was obtained in an effort to clone a receptor that binds kappa opioid receptor ligands (Xie, G.X., Proc Natl Acad Sci USA (1992) 89:4124-4128). A DNA molecule encoding a G-coupled receptor from a placental cDNA library was isolated. This receptor has an extremely high homology with the neurokinin B receptor (84% identical throughout the proposed protein sequence). When this clone was expressed in COS cells, it displayed opioid peptide displaceable

binding of ^{3}H-bremazocine (an opiate ligand with high affinity for kappa receptors). However, the low affinity of this receptor for ^{3}H-bremazocine, and the lack of appropriate selectivity since this receptor (binding both mu and delta ligands) made it doubtful that this cloned molecule is actually an opioid receptor.

Furthermore, characterization of opioid receptor proteins has proven difficult because of their instability once solubilized from the membrane; purified delta opioid receptors have not been isolated. The previous estimates of opioid receptor molecular weights ranging from 30 kDa to 60 Kda further reflect the difficulty in isolating and characterizing these proteins.

Recently, DNA encoding the murine kappa and delta opioid receptors from mouse brain was reported by Yasuda, K. *et al.* Proc Natl Acad Sci USA (1993) 90:6736-6740. The sequence of the clones indicated the presence of the expected seven transmembrane regions. In addition, Chen, Y. *et al.* in a soon-to-be-published manuscript in Molecular Pharmacology (1993) report the "molecular cloning and functional expression of a mu opioid receptor from rat brain". In fact, the rat mu receptor was cloned using the present inventors' DOR-1 clone, which lends enabling support to the present invention disclosed below. The mouse delta opioid receptor was disclosed as having been cloned (Kieffer, B.J. *et al.*, Proc Natl Acad Sci USA (1992) 89:12048-12052 (December issue) after the filing date of the priority document of the present application. However, the sequence reported therein differs from the sequence reported by the present inventors for the mouse

delta receptor (Evans *et al.*, 1992, *supra*; this disclosure).

In addition to the opioid receptors which respond to specified agonists, the delta, kappa and mu opioid receptors, additional forms of these proteins, commonly called opioid receptor-like (ORL) proteins have been obtained using the methods described herein. Using these methods, two human ORL protein-encoding cDNAs were obtained from a human brain stem cDNA library. One of these clones is equivalent to that isolated by O'Dowd, B.F. *et al.* Gene (1993) 136:355-360; the other, ORL-1, is identical to that reported by Mollereau, C. *et al.* FEBS Lett (1994) 341:33-38. A preliminary report of the present work appeared in Regulatory Peptides (1994) 54:143-144 and is incorporated herein by reference.

Disclosure of the Invention

The present invention provides recombinant nucleic acid molecules which encode the murine delta opioid receptor, as well as recombinant nucleic acid molecules which can be retrieved using low-stringency hybridization to this disclosed DNA. Thus, the invention provides genes encoding the delta, kappa and mu receptors, representing opioid receptors generally, including ORL proteins, of any species containing genes encoding such receptors or ORL proteins sufficiently homologous to hybridize under low-stringency conditions described herein.

As used herein, "opioid receptors" includes not only the previously identified delta, kappa and mu receptors, but also additional receptor-like proteins,

represented by, for example, ORL-1 that hybridize under the low-stringency conditions described to the murine DOR clone set forth herein, and which have opioid receptor characteristics including seven putative transmembrane regions, and ability to couple with guanine nucleotide-binding regulatory proteins (G proteins) to inhibit adenylyl cyclase and/or calcium channels or to stimulate potassium channels. Thus, when the word "opioid receptor" is used hereinbelow, this term is intended to include this entire genus.

Thus, in one aspect, the invention is directed to recombinant nucleic acid molecules and methods for the production of an opioid receptor wherein the opioid receptor is encoded by a gene which hybridizes under low-stringency to the nucleotide sequence of Figure 5 or to its complement. By "low-stringency" is meant 50% formamide/6 X SSC, overnight at 37°C for the hybridization, followed by washes at 2 X SSC 0.1% SDS at room temperature or 50% formamide at 37°C with washes of 1 X SSC at 37°C.

Also provided are expression systems comprising the nucleic acid molecules described above. The receptor can be recombinantly produced using these expression systems and host cells modified to contain them.

Especially useful are vertebrate cells which express the opioid receptor gene so that the opioid receptor protein is displayed at the surface of the cells. These cells offer means to screen native and synthetic candidate agonists and antagonists for the opioid receptors.

In still other aspects, the invention is directed to methods to screen candidate agonists and/or antagonists acting at opioid receptors using the recombinant transformed cells of the invention. Such assays include (1) binding assays using competition with ligands known to bind opioid receptors, (2) agonist assays which analyze activation of the secondary pathways associated with opioid receptor activation in the transformed cells, and (3) assays which evaluate the effect on binding of the candidate to the receptor by the presence or absence of sodium ion and GTP. Antagonist assays include the combination of the ability of the candidate to bind the receptor while failing to effect further activation, and, more importantly, competition with a known agonist.

Still another aspect of the invention is provision of antibody compositions which are immunoreactive with the opioid receptor proteins. Such antibodies are useful, for example, in purification of the receptors after solubilization or after recombinant production thereof.

In still other aspects, the invention is directed to probes useful for the identification of DNA which encodes related opioid receptors in various species or different types and subtypes of opioid receptors.

Accordingly, an object of the present invention is to provide an isolated and purified form of a DNA sequence encoding an opioid receptor, which is useful as a probe as well as in the production of the receptor.

Another object is to provide a recombinantly produced DNA sequence encoding an opioid receptor.

Another object is to produce an antisense sequences corresponding to known sense sequences encoding the opioid receptors.

Another object of the invention is to provide a DNA construct comprised of a control sequence operatively linked to a DNA sequence which encodes an opioid receptor and to provide recombinant host cells modified to contain the DNA construct.

Another object is to isolate, clone and characterize, from various vertebrate species, DNA sequences encoding the various related receptors, by hybridization of the DNA derived from such species with a native DNA sequence encoding the opioid receptor of the invention.

An advantage of the present invention is that opioid receptor-encoding DNA sequences can be expressed at the surface of host cells which can conveniently be used to screen drugs for their ability to interact with and/or bind to the receptors.

These and other objects, advantages and features of the present invention will become apparent to those persons skilled in the art.

Brief Description of the Drawings

Figure 1 depicts a comparison of binding of ^{3}H-diprenorphine (saturation curves) between NG108-15 cells and COS cells three days following transfection (by electroporation) of each with DOR-1 in the CDM-8 vector. Specific opioid binding was undetectable in nontransfected COS cells or COS cells transfected with plasmid alone.

Figure 2 depicts displacement curves of 5 nM ^{3}H-diprenorphine from COS cell membranes of cells transfected with DOR-1. ^{3}H-diprenorphine was displaced by diprenorphine, etorphine, morphine and levorphanol, but not by dextrorphan (the non-opiate active optical isomer of levorphanol).

Figure 3 depicts displacement curves of 5nm ^{3}H-diprenorphine from COS cell membranes of cells transfected with DOR-1. ^{3}H-diprenorphine was displaced by DPDPE and DSLET, which are delta-selective agonists, by DADLE, a high affinity ligand for mu and delta receptors, and by dynorphin 1-17, a kappa-preferring ligand. ^{3}H-diprenorphine was not displaced by DAGO, a mu-selective ligand.

Figure 4 depicts the results of a Northern analysis of mRNA from NG108-15 cells and cells from various rat brain regions.

Figure 5 shows the nucleotide sequence and the deduced amino acid sequence of the DOR-1 clone.

Figure 6 depicts the deduced amino acid sequence of DOR-1, compared with the rat somatostatin receptor. Consensus glycosylation sites predicted to fall in extracellular domains are indicated by an asterisk. Potential protein kinase C sites are listed in Example 5. The seven predicted membrane spanning regions (underlined) are predicted based on the hydrophobicity profile and published predictions (MacVector software program (IBI); T. Hopp, and K. Woods, Proc Natl Acad Sci USA (1981) 78:3842-3828). For sequencing, the cDNA insert was subcloned into pBluescript and both strands were sequenced from single-stranded DNA using Sequenase and Taq cycle

sequencing. For ambiguities due to compressions 7-deaza-dGTP replaced dGTP in the sequencing reactions and the products were resolved on formamide gels.

Figure 7 depicts a Southern blot of radiolabeled DOR-1 cDNA probe hybridized at high stringency to NG108-15, mouse, rat and human DNA cut with BamHI.

Figure 8a shows a partial nucleotide sequence of the human delta opioid receptor genomic clone H3 (also designated human DORa or hDORa).

Figure 8b shows a partial nucleotide sequence of the human kappa opioid receptor genomic clone H14 (also designated human KORa or hKORa).

Figure 8c shows a partial nucleotide sequence of the human mu opioid receptor genomic clone H20 (also designated human MORa or hMORa).

Figure 8d shows the nucleotide sequence of the CACACA repeat near the H20 DNA.

Figure 9 shows the nucleotide sequence of the murine mu-receptor clone DOR-2 also named mMOR-1 or mMOR-1α.

Figure 10 shows the homology of various receptor amino acid sequences.

Figure 11 shows the complete DNA sequence of the cDNA retrieved from a human brain stem cDNA library and comprising a nucleotide sequence encoding the opioid receptor ORL-1. This cDNA encodes a 370-amino acid opioid receptor protein.

Figure 12 shows a comparison of ORL-1 and ORL-2 amino acid sequences with various human and murine delta, kappa, and mu receptors. ORL-1 is a protein of 370 amino acids and is compared with human mu opioid receptor

(hMOR), human delta opioid receptor (hDOR) and murine kappa opioid receptor (mKOR).

Modes of Carrying Out the Invention

The invention provides DNA encoding mammalian opioid receptor protein and additional recombinant nucleic acids, expression vectors and methods useful for the production of these proteins. In addition, eucaryotic cells, such as COS cells, transformed with the recombinant molecules of the invention so as to express opioid receptor proteins at their surface are useful in screening assays to identify candidate opioid agonists and antagonists. In addition, antibodies may be raised to the recombinantly produced opioid receptor proteins. These antibodies are useful in immunoassays for said protein and in affinity purification thereof.

Recombinant Opioid Receptor

Illustrated hereinbelow is the obtention of a cDNA encoding a murine delta opioid receptor. The complete DNA sequence of the cDNA, and the amino acid sequence encoded thereby, are set forth herein in Figure 5. The availability of this cDNA permits the retrieval of the corresponding opioid receptor-encoding DNA from other vertebrate species. Accordingly, the present invention places within the possession of the art, recombinant molecules and methods for the production of cells expressing opioid receptors of various types and of various vertebrate species. Thus, the cDNA of Figure 5, or a portion thereof, may be used as a probe to identify that portion of vertebrate genomic DNA or cDNA which

encodes an opioid receptor protein. Illustrative methods used to prepare a genomic library and identify the opioid receptor-encoding genes are described for convenience hereinbelow. Also exemplified as illustrating the method of the invention is the retrieval of human ORL-1 from a brain stem cDNA library.

The DOR-1 clone described in Figure 5 is a cDNA clone corresponding to the murine delta opioid receptor. The present inventors found, and describe herein, that screening of a human genomic library under conditions of low stringency results in the recovery of DNA encoding all three types of human opioid receptors. Similarly, a murine genomic clone was obtained. In addition, a cDNA clone was obtained from a mouse brain library encoding the murine mu opioid receptor. Thus, either cDNA libraries from appropriate sources, such as brain, or genomic libraries, are fruitful sources or substrates for obtaining the DNA of the present invention and the corresponding recombinant materials. The invention is thus directed to DNA encoding an opioid receptor of a vertebrate, wherein the opioid receptor is encoded by a nucleotide sequence which hybridizes under conditions of low stringency to the nucleotide sequence shown in Figure 5 or to its complement.

In the alternative, the DNA of Figure 5 or a portion thereof may be used to identify specific tissues or cells which express opioid receptor protein by analyzing the mRNA, for example, using Northern blot techniques. Those tissues which are identified as containing mRNA encoding opioid receptor protein using the probes of the invention are then suitable sources for

preparation of cDNA libraries which may further be probed using the cDNA described hereinbelow.

The DNA encoding the various vertebrate opioid receptor proteins, obtained in general as set forth above, according to the standard techniques described hereinbelow, can be used to produce cells which express the opioid receptor at their surface; such cells are typically eucaryotic cells, in particular, mammalian cells such as COS cells or CHO cells. Suitable expression systems in eucaryotic cells for such production are described hereinbelow. The opioid receptor proteins may also be produced in procaryotes or in alternative eucaryotic expression systems for production of the protein *per se*. The DNA encoding the protein may be ligated into expression vectors preceded by signal sequences to effect its secretion, or may be produced intracellularly, as well as at the cell surface, depending on the choice of expression system and host. If desired, the opioid receptor protein thus recombinantly produced may be purified using suitable means of protein purification, and, in particular, by affinity purification using antibodies or fragments thereof immunospecific for the opioid receptor protein.

The reader is reminded that the term "opioid receptor" as used herein includes not only the conventional delta, kappa and mu opioid receptors, but also opioid receptor-like proteins which interact with G proteins in a similar manner. These receptor-like proteins are useful in analogous ways, and offer additional screening tools for candidate compounds that

affect the central nervous system. They are thus useful for the same purposes as the "conventional" receptors.

Screening for Opioid Agonists and Antagonists Using Recombinant Cells

The ability of a candidate compound to act as an opioid agonist or antagonist may be assessed using the recombinant cells of the invention in a variety of ways. To exhibit either agonist or antagonist activity, the candidate compound must bind to the opioid receptor. Thus, to assess the ability of the candidate to bind, either a direct or indirect binding assay may be used. For a direct binding assay, the candidate binding compound is itself detectably labeled, such as with a radioisotope or fluorescent label, and binding to the recombinant cells of the invention is assessed by comparing the acquisition of label by the recombinant cells to the acquisition of label by corresponding untransformed (control) cells.

More convenient, however, is the use of a competitive assay wherein the candidate compound competes for binding to the recombinant cells of the invention with a detectably labeled form of an opioid ligand known to bind to the receptor. Such ligands are themselves labeled using radioisotopes or fluorescent moieties, for example. A particularly suitable opioid known to bind to this receptor is diprenorphine. A typical protocol for such an assay is as follows:

In general, about 10^6 recombinant cells are incubated in suspension in 1.0 ml of Kreb's Ringer Hepes Buffer (KRHB) at pH 7.4, 37°C for 20 min with ^{3}H-diprenorphine. Nonspecific binding is determined by

the addition of 400 nM diprenorphine in the binding mixtures. Various concentrations of candidate compounds are added to the reaction mixtures. The incubations are terminated by collecting the cells on Whatman GF-B filters, with removal of excess radioactivity by washing the filters three times with 5 ml of KRHB at 0°C. After incubating at 20°C overnight in 5 ml of scintillation fluid, such as Liquiscint (National Diagnostics, Somerville, NJ), the radioactivity on the filters is determined by liquid scintillation counting.

The K_d (dissociation constant) values for the candidate opiate ligands can be determined from the IC_{50} value ("inhibitory concentration$_{50}$" means the concentration of candidate ligand that results in a 50% decrease in binding of labeled diprenorphine).

The effects of sodium and GTP on the binding of ligands to the recombinantly expressed receptors can be used to distinguish agonist from antagonist activities. If the binding of a candidate compound is sensitive to Na^+ and GTP, it is more likely to be an agonist than an antagonist, since the functional coupling of opioid receptors to second messenger molecules such as adenylate cyclase requires the presence of both sodium and GTP (Blume et al., Proc Natl Acad Sci USA (1979) 73:26-35). Furthermore, sodium, GTP, and GTP analogues have been shown to effect the binding of opioids and opioid agonists to opioid receptors (Blume, Life Sci (1978) 22:1843-52). Since opioid antagonists do not exhibit binding that is sensitive to guanine nucleotides and sodium, this effect is used as a method for distinguishing agonists from antagonists using binding assays.

In addition, agonist activity can directly be assessed by the functional result within the cell. For example, it is known that the binding of opioid agonists inhibits cAMP formation, inhibits potassium channel activation, inhibits calcium channel activation, and stimulates GTPase. Assessment of these activities in response to a candidate compound is diagnostic of agonist activity. In addition, the ability of a compound to interfere with the activating activity of a known agonist such as etorphine effectively classifies it as an antagonist.

In one typical assay, the measurement of cAMP levels in cells expressing opioid receptors is carried out by determining the amount of ^{3}H-cAMP formed from intracellular ATP pools prelabeled with ^{3}H-adenine (Law *et al.*, *supra*). Thus, cAMP formation assays are carried out with 0.5 x 10^{6} cells/0.5 ml of KRHB at pH 7.4, incubated at 37°C for 20 minutes. After addition of the internal standard ^{32}P-cAMP, the radioactive cAMP is separated from other ^{3}H-labeled nucleotides by known double-column chromatographic methods. The opiate agonists' ability to inhibit cAMP accumulation is then determined as described by Law *et al.* (*supra*).

The potency of a candidate opiate antagonist can be determined by measuring the ability of etorphine to inhibit cyclic AMP accumulation in the presence and in the absence of known amounts of the candidate antagonist. The inhibition constant (K_i) of an antagonist can then be calculated from the equation for competitive inhibitors.

An interesting feature of screening assays using the prior art NG108-15 cells is that the agonist adenylate

cyclase inhibition function apparently does not require binding of all receptors on these cells. Thus, the K_d and K_i values for the opioid ligands differed when using these cells.

The foregoing assays, as described above, performed on the recombinantly transformed cells of the present invention, provide a more direct and more convenient screen for candidate compounds having agonist and antagonist opioid receptor activity than that previously available in the art. Furthermore, such assays are more sensitive since cells can, in accordance with the present invention, be engineered to express high levels of the opioid receptor. Additionally, cells engineered in accordance with the present invention will circumvent the concern that NG108-15 cells, due to their tumor cell background, have a cellular environment that artifactually affects opioid receptor expression.

The mu opioid encoding DNA described herein also offer a means to follow inheritance patterns. DNA sequence polymorphisms frequently occur in the noncoding regions that surround genes. Polymorphisms are especially frequent in repeat sequences such as CACACA which often show distinct polymorphisms in the number of repeats that are present in different individuals. These polymorphisms offer a marker by which to follow the inheritance of the gene among family members. The inheritance of a gene (such as MORa) or its human counterpart can be followed by polymerase chain reaction (PCR) amplification of the region surrounding the CACACA polymorphism and analyzing the resulting products. This would be a useful diagnostic marker for the mu opioid receptor gene.

Methods to Prepare Opioid Receptor Protein or Portions Thereof

The present invention provides the amino acid sequence of a murine opioid receptor; similarly, the availability of the cDNA of the invention places within possession of the art corresponding vertebrate opioid receptors whose amino acid sequence may also be determined by standard methods. As the amino acid sequences of such opioid receptors are known, or determinable, in addition to purification of such receptor protein from native sources, recombinant production or synthetic peptide methodology may also be employed for producing the receptor protein or peptide.

The opioid receptor or portions thereof can thus also be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, the DNA encoding these peptides may be synthesized using commercially available oligonucleotide synthesis instrumentation for production of the protein in the manner set forth above. Production using solid phase peptide synthesis is, of course, required if amino acids not encoded by the gene are to be included.

The nomenclature used to describe the peptides and proteins of the invention follows the conventional practice where the N-terminal amino group is assumed to be to the left and the carboxy group to the right of each amino acid residue in the peptide. In the formulas representing selected specific embodiments of the present invention, the amino- and carboxy-terminal groups, although often not specifically shown, will be understood to be in the form they would assume at physiological pH

values, unless otherwise specified. Thus, the N-terminal $NH3^+$ and C-terminal COO^- at physiological pH are understood to be present though not necessarily specified and shown, either in specific examples or in generic formulas. Free functional groups on the side chains of the amino acid residues may also be modified by glycosylation, phosphorylation, cysteine binding, amidation, acylation or other substitution, which can, for example, alter the physiological, biochemical, or biological properties of the compounds without affecting their activity within the meaning of the appended claims.

In the peptides shown, each gene-encoded residue, where appropriate, is represented by a single letter designation, corresponding to the trivial name of the amino acid, in accordance with the following conventional list:

Amino Acid	One-Letter Symbol	Three-letter Symbol
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamine	Q	Gln
Glutamic acid	E	Glu
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

Nomenclature of Enkephalins

Enkephalins are either of two peptides having five residues with the N-terminal residue numbered 1:

tyr-gly-gly-phe-xxx
1 2 3 4 5

In "met enkephalin" the fifth residue is methionine:

tyr-gly-gly-phe-met

In "leu enkephalin" the 5th residue is leucine:

tyr-gly-gly-phe-leu

Enkephalin analogs can be made with (1) amino acid substitutions, (2) D-amino acid substitutions, and/or (3) additional amino acids. The site at which the substitution is made is noted at the beginning of the compound name. For example, "(D-ala^2, D-leu^5) enkephalin"

means that D-ala is present at the second position and D-leu is present at the fifth position:

tyr-[D-ala]-gly-phe-[D-leu]

One letter abbreviations can also be used. Thus, "(D-ser^2) leu enkephalin" could be abbreviated as "DSLE." Additional residues are noted as well. Thus, the addition of a threonine residue (to the sixth position) of (D-ser^2) leu enkephalin would be "(D-ser^2) leu enkephalin thr" which could be abbreviated as "DSLET":

tyr-[D-ser]-gly-phe-leu-thr

Antibodies

Antibodies immunoreactive with the opioid receptor protein or peptide of the present invention can be obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions those portions of the receptor intended to be targeted by the antibodies. Certain protein sequences have been determined to have a high antigenic potential. Such sequences are listed in antigenic indices, for example, MacVector software (I.B.I.) Thus, by determining the sequence of the opioid receptor protein and evaluating the sequence with an antigenic index, probable antigenic sequences are located.

Antibodies are prepared by immunizing suitable mammalian hosts according to known immunization protocols using the peptide haptens alone, if they are of sufficient length, or, if desired, or if required to enhance immunogenicity, conjugated to suitable carriers. Methods for preparing immunogenic conjugates with carriers such as BSA, KLH, or other carrier proteins are well known in the

art. In some circumstances, direct conjugation using, for example, carbodiimide reagents may be effective; in other instances linking reagents such as those supplied by Pierce Chemical Co., Rockford, IL, may be desirable to provide accessibility to the hapten. The hapten peptides can be extended or interspersed with cysteine residues, for example, to facilitate linking to carrier. Administration of the immunogens is conducted generally by injection over a suitable time period and with use of suitable adjuvants, as is generally understood in the art. During the immunization schedule, titers of antibodies are taken to determine adequacy of antibody formation.

While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, use of monoclonal antibody (mAb) preparations is preferred. Immortalized cell lines which secrete the desired mAbs may be prepared using the standard method of Kohler and Milstein or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known. The immortalized cell lines secreting the desired mAbs are screened by immunoassay in which the antigen is the peptide hapten or is the opioid receptor itself displayed on a recombinant host cell. When the appropriate immortalized cell culture secreting the desired mAb is identified, the cells can be cultured either *in vitro* or by intraperitoneal injection into animals wherein the mAbs are produced in the ascites fluid.

The desired mAbs are then recovered from the culture supernatant or from the ascites fluid. In addition to intact antibodies, fragments of the mAbs or of

polyclonal antibodies which contain the antigen-binding portion can be used as antagonists. Use of immunologically reactive antigen binding fragments, such as the Fab, Fab', of $F(ab')_2$ fragments, is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin molecule.

Standard Methods

The techniques for sequencing, cloning and expressing DNA sequences encoding the amino acid sequences corresponding to a opioid receptor, *e.g.*, polymerase chain reaction (PCR), synthesis of oligonucleotides, probing a cDNA library, transforming cells, constructing vectors, preparing antisense oligonucleotide sequences based on known sense nucleotide sequences, extracting messenger RNA, preparing cDNA libraries, and the like are well-established in the art. Ordinarily skilled artisans are familiar with the standard resource materials, specific conditions and procedures. The following paragraphs are provided for convenience, it being understood that the invention is limited only by the appended claims.

RNA Preparation and Northern Blot

RNA preparation is as follows: The samples used for preparation of RNA are immediately frozen in liquid nitrogen and then stored until use at -80°C. The RNA is prepared by CsCl centrifugation (Ausubel et al., *supra*) using a modified homogenization buffer (Chirgwin et al., Biochemistry (1979) 18:5294-5299). Poly(A^+) RNA is selected by oligo(dT) chromatography (Aviv and Leder, Proc

Natl Acad Sci USA (1972) 69:1408-1412). RNA samples are stored at -80°C.

Analysis of gene expression and tissue distribution can be accomplished using Northern blots with, *e.g.*, radiolabeled probes. The mRNA is size-separated using gel electrophoresis and then typically is transferred to a nylon membrane or to nitrocellulose and hybridized with radiolabeled probe. Presence of the hybridized probe is detected using autoradiography.

Cloning

The cDNA sequences encoding the opioid receptor protein are obtained from a random-primed, size-selected cDNA library.

Alternatively, the cDNA sequences encoding opioid receptor protein are obtained from a cDNA library prepared from mRNA isolated from cells expressing the receptor protein in various organs such as the brain, according to procedures described in Sambrook, J. *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989.

The cDNA insert from the successful clone, excised with a restriction enzyme such as EcoRI, is then used as a probe of the original cDNA library or other libraries (low stringency) to obtain the additional clones containing inserts encoding other regions of the protein that together or alone span the entire sequence of nucleotides coding for the protein.

An additional procedure for obtaining cDNA sequences encoding the opioid receptor protein is PCR.

PCR is used to amplify sequences from a pooled cDNA library of reversed-transcribed RNA, using oligonucleotide primers based on the transporter sequences already known.

Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employs ligation and restriction techniques which are well understood in the art (Young et al., Nature (1988) 316:450-452). Double-stranded cDNA encoding opioid receptor protein is synthesized and prepared for insertion into a plasmid vector CDM8. Alternatively, vectors such as Bluescript² or Lambda ZAP² (Stratagene, San Diego, CA) or a vector from Clontech (Palo Alto, CA) can be used in accordance with standard procedures (Sambrook, J. *et al.*, *supra*).

Site specific DNA cleavage is performed by treating with the suitable restriction enzyme, such as EcoRI, or more than one enzyme, under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, *e.g.*, New England Biolabs, Product Catalog. In general, about 1 μg of DNA is cleaved by one unit of enzyme in about 20 μl of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to ensure complete digestion of the DNA substrate. Incubation times of about one to two hours at about 37°C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and can be followed by other extraction and the nucleic

acid recovered from aqueous fractions by precipitation with ethanol.

In vector construction employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIP) in order to remove the 5' phosphate and prevent religation of the vector. Digestions are conducted at pH 8 in approximately 150 mM Tris, in the presence of Na^+ and Mg^{++} using about 1 unit of BAP or CIP per μg of vector at 60°C or 37°C, respectively, for about one hour. In order to recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol precipitated. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments.

Ligations are performed in 15-50 μl volumes under the following standard conditions and temperatures: 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 33 μg/ml BSA, 10 mM to 50 mM NaCl, and either 40 μM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 μg/ml total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt end ligations (usually employing a 10-30 fold molar excess of linkers) are performed at 1 μM total ends concentration. Correct ligations for vector construction are confirmed according to the procedures of Young et al., *supra*.

cDNA Library Screening

cDNA libraries can be screened using reduced stringency conditions as described by Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing and Wiley-Interscience, New York (1990), or by using methods described in Sambrook et al. *supra*), or by using a colony or plaque hybridization procedure with a fragment of the DOR-1 cDNA coding for opioid receptor protein.

Plaque hybridization is typically carried out as follows: Host bacteria such as LE 392 (Stratagene) are grown overnight at 37° in LB Broth (Sambrook *et al.*, *supra)*, gently pelleted and resuspended in one half the original volume of 10 mM $MgSO_4$, 10 mM $CaCl_2$. After titration, an amount of the phage library containing approximately 50,000 plaque forming units (pfu) is added to 300 μl of the host bacteria, incubated at 37° for 15 minutes and plated onto NZYCM agar with 10 ml NZYCM top agarose. A total of a million plaques distributed on twenty 15 cm plates are screened. For colony screening, transfected bacteria are plated onto LB broth plates with the appropriate antibiotics. After the plaques or colonies have grown to 1 mm, the plates are chilled at 4°C for at least two hours, and then overlaid with duplicate nitrocellulose filters, followed by denaturation of the filters in 0.5 M NaOH/1.5 M NaCl for five minutes and neutralization in 0.5 M Tris, pH 7.4/1.5 M NaCl for five minutes. The filters are then dried in air, baked at 80°C for two hours, washed in 5X SSC/0.5% SDS at 68°C for several hours, and prehybridized in 0.5 M $NaPO_4$, pH 7.2/1% BSA/1 mM EDTA/7% SDS/100 μg/ml denatured salmon sperm DNA for more than 4 hours. Using the DOR-1 cDNA (described

herein) labeled by random priming as a probe, high stringency hybridization is carried out in the same solution at 68°C, and the temperature is reduced to 50-60°C for lower stringency hybridization. After hybridization for 16-24 hours, the filters are washed first in 40 mM $NaPO_4$, pH 7.2/0.5% BSA/5% SDS/1 mM EDTA twice for one hour each, then in 40 mM $NaPO_4$, pH 7.2/1% BSA/1 mM EDTA for one hour each, both at the same temperature as the hybridization (Boulton et al., Cell (1991) 65:663-675). The filters are then exposed to film with an enhancing screen at -70°C for one day to one week.

Positive signals are then aligned to the plates, and the corresponding positive phage is purified in subsequent rounds of screening, using the same conditions as in the primary screen. Purified phage clones are then used to prepare phage DNA for subcloning into a plasmid vector for sequence analysis. Tissue distribution of DNA corresponding to the various independent clones is analyzed using Northern blots and *in situ* hybridization using standard methods. Function of the DNA is tested using expression in a heterologous eucaryotic expression system such as COS cells.

Expression of Opioid Receptor Protein

The nucleotide sequence encoding opioid receptor protein can be expressed in a variety of systems. The cDNA can be excised by suitable restriction enzymes and ligated into procaryotic or eucaryotic expression vectors for such expression.

For example, as set forth below, the cDNA encoding the protein is expressed in COS cells. To effect

functional expression, the plasmid expression vector CDM8 (Aruffo and Seed, Proc Natl Acad Sci USA (1987) 84:8573-8577, provided by Drs. Aruffo and Seed (Harvard University, Boston, MA) was used. Alternatively, other suitable expression vectors such as retroviral vectors can be used.

Procaryotic and preferably eucaryotic systems can be used to express the opioid receptor. Eucaryotic microbes, such as yeast, can be used as hosts for mass production of the opioid receptor protein. Laboratory strains of *Saccharomyces cerevisiae*, Baker's yeast, are used most, although a number of other strains are commonly available. Vectors employing, for example, the 2μ origin of replication (Broach, Meth Enz (1983) 101:307), or other yeast compatible origins of replications (*e.g.*, Stinchcomb et al., Nature (1979) 282:39); Tschempe et al., Gene (1980) 10:157; and Clarke et al., Meth Enz (1983) 101:300) can be used. Control sequences for yeast vectors include promoters for the synthesis of glycolytic enzymes (Hess et al., J Adv Enzyme Reg (1968) 7:149; Holland et al., Biochemistry (1978) 17:4900). Additional promoters known in the art include the promoter for 3-phosphoglycerate kinase (Hitzeman et al., J Biol Chem (1980) 255:2073), and those for other glycolytic enzymes. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and enzymes responsible for maltose and galactose utilization. It is also believed terminator sequences are

desirable at the 3' end of the coding sequences. Such terminators are found in the 3' untranslated region following the coding sequences in yeast-derived genes.

Alternatively, genes encoding opioid receptor protein are expressed in eucaryotic host cell cultures derived from multicellular organisms. (See, e.g., Tissue Cultures, Academic Press, Cruz and Patterson, eds, (1973)). These systems have the additional advantage of the ability to splice out introns, and thus can be used directly to express genomic fragments. Useful host cell lines include amphibian oocytes such as *Xenopus* oocytes, COS cells, VERO and HeLa cells, Chinese hamster ovary (CHO) cells, and insect cells such as SF9 cells. Expression vectors for such cells ordinarily include promoters and control sequences compatible with mammalian cells such as, for example, the commonly used early and late promoters from baculovirus, vaccinia virus, Simian Virus 40 (SV40) (Fiers et al., Nature (1973) 273:113), or other viral promoters such as those derived from polyoma, Adenovirus 2, bovine papilloma virus, or avian sarcoma viruses. The controllable promoter, hMTII (Karin et al., Nature (1982) 299:797-802) may also be used. General aspects of mammalian cell host system transformations have been described by Axel, U.S. Patent No. 4,399,216. It now appears, that "enhancer" regions are important in optimizing expression; these are, generally, sequences found upstream or downstream of the promoter region in non-coding DNA regions. Origins of replication can be obtained, if needed, from viral sources. However, integration into the chromosome is a common mechanism for DNA replication in eucaryotes.

If procaryotic systems are used, an intronless coding sequence should be used, along with suitable control sequences. The cDNA of opioid receptor protein can be excised using suitable restriction enzymes and ligated into procaryotic vectors along with suitable control sequences for such expression.

Procaryotes most frequently are represented by various strains of *E. coli*; however, other microbial species and strains may also be used. Commonly used procaryotic control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, including such commonly used promoters as the β-lactamase (penicillinase) and lactose (lac) promoter systems (Chang et al., Nature (1977) 198:1056) and the tryptophan (trp) promoter system (Goeddel et al., Nucl Acids Res (1980) 8:4057) and the λ derived P_L promoter and N-gene ribosome binding site (Shimatake et al., Nature (1981) 292:128).

Depending on the host cell used, transformation is carried out using standard techniques appropriate to such cells. The treatment employing calcium chloride, as described by Cohen, Proc Natl Acad Sci USA (1972) 69:2110 (1972) or by Sambrook *et al.* (*supra*), can be used for procaryotes or other cells which contain substantial cell wall barriers. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology (1978) 54:546, optionally as modified by Wigler et al., Cell (1979) 16:777-785, or by Chen and Okayama, *supra*, can be used. Transformations into yeast can be carried out according to the method of

Van Solingen et al., J Bact (1977) 130:946, or of Hsiao et al., Proc Natl Acad Sci USA (1979) 76:3829.

Other representative transfection methods include viral transfection, DEAE-dextran mediated transfection techniques, lysozyme fusion or erythrocyte fusion, scraping, direct uptake, osmotic or sucrose shock, direct microinjection, indirect microinjection such as via erythrocyte-mediated techniques, and/or by subjecting host cells to electric currents. The above list of transfection techniques is not considered to be exhaustive, as other procedures for introducing genetic information into cells will no doubt be developed.

Modulation of Expression by Antisense Sequences

Alternatively, antisense sequences may be inserted into cells expressing opioid receptors as a means to modulate functional expression of the receptors encoded by sense oligonucleotides. The antisense sequences are prepared from known sense sequences (either DNA or RNA), by standard methods known in the art. Antisense sequences specific for the opioid receptor gene or RNA transcript can be used to bind to or inactivate the oligonucleotides encoding the opioid receptor.

Terminology

As used herein, the singular forms "a", "an" and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a receptor" includes mixtures of such receptors, reference to "an opioid" includes a plurality of and/or mixtures of such opioids and reference to "the host cell" includes a

plurality of such cells of the same or similar type and so forth.

Unless defined otherwise all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The following examples are intended to illustrate but not to limit the invention. Temperatures are in °C and pressures at near atmospheric unless otherwise specified.

Preparation of Mono ^{125}I-DADLE

DADLE (Peninsula Laboratories Inc.) was iodinated using the iodogen method (Maidment et al., in: MICRODIALYSIS IN THE NEUROSCIENCES, T. Robinson and J. Justice, eds., pp. 275-303 (Elsevier, 1991)). Both mono- and di-iodinated forms are produced. It has been reported that di-iodo-DADLE does not bind opiate receptors, due to the di-iodination of the tyrosine residue (Miller, R.J., et al., Life Sci (1978) 22:379-88). Accordingly, mono-iodinated DADLE is preferred. Mono-^{125}I-DADLE is also preferred because it has extremely high specific activity compared to DADLE labeled with other isotopes. Thus, exposure times on the order of days, rather than weeks or months can be used.

By employing a molar ratio of sodium iodide to peptide of approximately 1:100 when carrying out iodination, the yield of the preferred mono-iodinated DADLE was increased. Additionally, to further enhance the yield of the mono-iodinated form, iodinated DADLE (containing both mono- and di-iodinated forms) was purified by reverse-phase HPLC (Maidment et al., *supra*).

Employing this procedure a single major radiolabeled peak of the mono-iodinated DADLE separated from di-iodinated and non-iodinated forms.

DADLE monolabeled with ^{125}I is crucial to successful screening. Radiolabeled ^{125}I-DADLE differs from DADLE in several important parameters: size, hydrophobicity, and binding affinity (slightly lower). The purification of mono-iodinated from di-iodinated and non-iodinated DADLE by the HPLC step yields a ligand with very high specific activity (approximately 2000 Ci/mmol). The specific activity of the mono-iodinated form is approximately 100 times greater than that obtained by using the unseparated mixture of mono-, di-, and non-iodinated DADLE. Monolabeled ^{125}I-DADLE must be used within a few days of its preparation.

Example 1

Preparation of DOR-1

The NG108-15 cell line (available from Dr. Christopher Evans, UCLA) comprises a homogeneous and enriched source of delta opioid receptors. Utilizing mRNA isolated from NG108-15, a random-primed, size-selected cDNA library was constructed in plasmid vector CDM8. The cDNA library was amplified in bacteria. The cDNA library was transfected into COS-7 cells by electroporation. Transiently transfected COS lawns were screened and selected with highly purified mono-^{125}I-2dAla, 5dLeu enkephalin (^{125}I-DADLE). Positive clones were identified by film autoradiography, and plasmids from these cells were recovered and amplified in bacteria. Thereafter, the plasmids were re-transfected into COS cells. Following

three cycles of such plasmid enrichment, individual clones were transfected and a pure clone was identified that bound ^{125}I-DADLE.

A. Construction of the cDNA Library

RNA was prepared from NG108-15 cells by homogenization in 6 M guanidinium isothiocyanate, followed by centrifugation through cesium chloride (J.M. Chirgwin, et al., Biochemistry (1979) 18:5294). Poly-A$^+$ RNA was isolated by chromatography over oligo-dT-cellulose (H. Aviv and P. Leder, Proc Natl Acad Sci USA (1972) 69:1408). Using this RNA as a template, random hexamers were used to prime cDNA synthesis by avian myeloblastosis virus reverse transcriptase (Life Sciences Inc.). Second strand synthesis was accomplished with RNase-H and *E. coli* DNA polymerase (U. Gubler and B.J. Hoffman, Gene (1983) 24:263). The ends of the cDNAs were rendered blunt with T4 DNA polymerase and BstXI linkers were added. cDNA longer than 1.5kb was selected by electrophoresis through 5% acrylamide followed by electro-elution. The 1.5kb cDNA was ligated to the CDM8 vector (A. Aruffo and B. Seed, *supra*, and then transformed into MC-1061 bacteria by electroporation (W.J. Dower *et al.*, Nucl Acids Res (1988) 16:6127). Accordingly, six pools of plasmid DNA were prepared from the original cDNA library of approximately $2x10^6$ recombinants.

B. Plasmid Transfection by Electroporation and Expression in COS cells

COS cells were grown at high density and were harvested in trypsin, then resuspended at $2x10^7$/ml in 1:2X RPMI containing 20% fetal calf serum. These cells were then incubated for ten minutes at 4°C with 20 μg recombinant plasmid DNA from the cDNA library described above, and then electroporated at 960 μF and 230 V in a 0.4 cm gap cuvette (BioRad). The cells were then incubated an additional ten minutes at 4°C, and then plated into Dulbecco's Modified Eagle's Medium (DMEM) plus 10% fetal calf serum (FCS).

C. Screening of Transfected COS Cells

The transfected COS cells as obtained above were grown for three days, then screened using radiolabeled mono ^{125}I-DADLE. Transfected COS lawns were washed with PBS, then incubated at room temperature with 10-20 nM ^{125}I-DADLE in KHRB containing 1% BSA. After 1 hour, the plates were washed rapidly several times with ice cold PBS then dried on ice with strong flow of forced cold air. Plates were exposed on Dupont Cronex film in cassettes at room temperature. Positive clones were identified by careful alignment of the film with the petri dish via low power microscopy.

DNA was removed from positive cells by solubilization in 0.1% SDS in TE containing 1 μg/μl tRNA delivered from a syringe attached to a capillary tube on a micromanipulator. Plasmids were purified from the extracted cells using the Hirt lysis procedure (Hirt, B., J Mol Biol (1967) 26:365-369), and electroporated into MC-

1061 bacteria. The plasmids were purified then retransfected into COS cells. Following three such enriching cycles, individual plasmid clones were transfected into COS cells yielding a single clone, named the DOR-1 clone.

Example 2

Characterization of DOR-1

The DOR-1 clone initially was characterized by screening cell membrane fractions, from cells expressing DOR-1, with the labelled DADLE it was found that binding of ^{125}I DADLE was displaced by nanomolar concentrations of opiate alkaloids diprenorphine, morphine, etorphine, and by DADLE, DSLET and DPDPE. Dextrorphan (10μM) did not displace the ^{125}I DADLE, whereas its opioid-active enantiomer levorphanol did displace the radiolabeled DADLE. Additionally, the mu receptor-selective ligand DAGO (5μM) did not displace the counts.

The DOR-1 clone was further characterized pharmacologically by assessing binding of ^{3}H-diprenorphine to intact cells expressing the DOR-1 clone (Figure 1), and by assessing displacement of ^{3}H-diprenorphine from membrane fractions of such cells (Figures 2 and 3).

Binding assays were conducted on intact cells in KRHB, 1% BSA; or on membranes in 25 mM HEPES, 5 mM $MgCl_2$ pH 7.7. Cells were harvested with PBS containing 1 mM EDTA, washed 2x with PBS then resuspended in KHRB. Membranes prepared from the cells (Law P.Y.E et al., Mol Pharm (1983) 23:26-35) were used directly in the binding assay. Binding assays were conducted in 96 well polypropylene cluster plates (Costar), at 4°C in a total

volume of 100μl with an appropriate amount of radiolabeled ligand. Following 1 hour of incubation, plates were harvested on a Tomtec harvester and "B" type filtermats were counted in a Betaplate (Pharmacia) scintillation counter using Meltilex B/HS (Pharmacia) melt-on scintillator sheets.

Intact cells expressing DOR-1 were analyzed with the high affinity opiate antagonist ^{3}H-diprenorphine. Specific binding was defined by the counts displaced by 400 nM diprenorphine. Figure 1 shows a saturation curve for ^{3}H-diprenorphine for NG108-15 cells, and COS-7 cells transfected with the delta opioid receptor clone. Untransfected COS cells, or COS cells transfected with plasmid having no insert showed no specific binding. Thus, the opioid binding of COS-DOR-1 cells was similar to that of NG108-15 cells.

Membranes prepared by standard methods from transfected COS-7 cells were employed for a more extensive pharmacological characterization of the receptor encoded by the DOR-1 clone. The affinities for the following alkaloid opiates in competition for ^{3}H-diprenorphine are illustrated in Figure 2: unlabeled diprenorphine, a high affinity antagonist for delta receptors; etorphine, a high affinity agonist for delta, mu and kappa receptors; levorphanol, a low affinity agonist for delta receptors; morphine, a low affinity agonist for delta receptors and a high affinity agonist for mu receptors; and dextrorphan, a non-opiate active enantiomer of levorphanol which should not bind delta receptors.

As shown in Figure 2, the displacement of ^{3}H-diprenorphine, in decreasing order of affinity, was

observed with diprenorphine, etorphine, levorphanol and morphine. As expected, ^{3}H-diprenorphine was not displaced by dextrorphan.

The affinities of the following opioid peptides in competition for ^{3}H-diprenorphine are set forth in Figure 3: DADLE, a high affinity agonist for mu and delta receptors; DSLET and DPDPE, both high affinity agonists of delta (but not mu) receptors; DAGO, a selective agonist for mu receptors; and Dynorphin 1-17, a high affinity agonist for kappa receptors and moderate to low affinity agonist for delta receptors. As shown in Figure 3, the displacement of ^{3}H-diprenorphine, in decreasing order of affinity, was observed for DSLET, DPDPE and DADLE, and Dynorphin 1-17. Only weak displacement by DAGO was observed.

Example 3

Northern Blot Analysis of RNA

For Northern analysis, the mRNA from NG108-15 cells, and from cells dissected from regions of rat brain was separated by electrophoresis through 2.2 M formaldehyde/1.5% agarose, blotted to nylon and hybridized in aqueous solution at high stringency. The filters were prehybridized in 0.5 M $NaPO_4$, pH 7.2; 1% BSA; 1 mM EDTA; 7% SDS; and 100 μg/ml denatured salmon sperm DNA for at least four hours at 68°C (Boulton *et al.*, *supra*). The filters were then hybridized overnight under these same conditions with $\geq$ 5x 10^6 cpm/ml purified cDNA insert labelled by random priming (A.P. Feinberg and B. Vogelstein, Anal Biochem (1983) 132:6). The filters were twice washed in 40 mM $NaPO_4$, pH 7.2; 0.5% BSA; 5% SDS; and

1 mM EDTA for one hour, and then washed twice in 40 mM $NaPO_4$, pH 7.2; 1% SDS; and 1 mM EDTA for one hour each, all at 68°C. Thereafter autoradiography was performed with DuPont Cromex Lightening Plus at -70°C.

The results of the Northern analysis of the mRNA showed the presence of multiple bands hybridizing to the probe at approximately 8.7, 6.8, 4.4, 2.75 and 2.2 kilobases (Kb) (Figure 4). Also, the Northern analysis indicates that the pattern of mRNA may vary between brain regions. At present, it is unclear whether these mRNAs encode different protein sequences, and if so, whether these messages represent different types or sub-types of opioid receptors.

Example 4

Southern Blot Analysis of DNA

The radiolabeled DOR-1 cDNA probe was hybridized to genomic Southern blots by standard methods (Sambrook *et al.*, *supra*). Accordingly, the radiolabeled DOR-1 cDNA probe was hybridized under high stringency conditions to a blot of NG108-15, mouse, rat and human DNA cut with restriction endonuclease BamHI (Figure 7). Single bands were observed in the clones containing the NG108-15, mouse, and rat DNA. The sizes of the bands hybridizing to the cDNA probe were estimated to be 5.2kb (NG108-15), 5.2kb (mouse), and 5.7kb (rat). These results indicate the close homology of the mouse and rat genes, and also demonstrate that the DOR-1 clone is from the murine parent of the NG108-15 cell line.

In a blot containing EcoRI-cut genomic DNA from many different species, hybridization of the DOR-1 cDNA

under conditions of moderate stringency showed two bands in each lane of mouse, rat, human, rabbit, and several other mammalian species. This demonstrates a close relationship between opioid receptor genes in all of these species. Further, these results show that the genes or cDNAs from each of these species may readily be cloned using hybridization under moderate stringency.

Example 5

Determination of the cDNA Sequence

Isolated cDNA represented by the DOR clone was analyzed by subcloning the insert from the cDNA clone into a plasmid such as pBluescript™ (Stratagene, San Diego, CA) and using the dideoxy method (Sanger et al., Proc Natl Acad Sci USA (1977) 74:5463-5467). The sequence of the cDNA was determined from single-stranded DNA and specifically designed internal primers, using both Sequenase and ΔTaq cycle sequencing kits (USB). These kits, widely used in the art, utilize the dideoxy chain termination method. The DNA sequence and predicted protein sequence was then compared to sequences in established databanks such as GenBank.

Sequencing the cDNA insert in the DOR-1 clone, revealed an open reading frame of 370 amino acids (Figure 5). Comparisons with known sequences in GenBank showed highest homology between DOR-1 and the G-protein-coupled somatostatin receptor (57% amino acid identity), and slightly lower homology with the receptors binding angiotensin, the two chemotactic factors IL-8 and N-formyl peptide. Figure 6 shows the homology to the human somatostatin 1 receptor. The close homology of the

present receptor clone with the somatostatin receptor is especially noteworthy since somatostatin ligands are reported to bind to opioid receptors, and to have molecular mechanisms similar to those in delta receptors.

Other features of the DOR-1 clone amino acid sequence deduced from the cDNA sequence include three consensus glycosylation sites at residues 18 and 33 (predicted to be in the extracellular N-terminal domain), and at residue 310 (close to the C-terminus and predicted to be intracellular). Phosphokinase C consensus sites are present within predicted intracellular domains, at residues 242, 255, 344, and 352. Seven putative membrane-spanning regions were identified based on hydrophobicity profiles, as well as homology with Rhodopsin and other G-protein coupled receptors which have been analyzed with respect to membrane-spanning regions using MacVector (I.B.I.) analysis. The DOR-1 clone isolated in accordance with the principles of the present invention produces a delta receptor with a predicted molecular weight of 40,558 daltons prior to post-translational modifications such as N-glycosylation.

Example 6

Isolation of Opioid Receptor Genomic Clones

Isolation of genomic clones was carried out according to techniques known in the art. To isolate opiate receptor genomic clones, 300,000 human genomic clones in γgem 11 (Promega) and a similar number of mouse genomic clones in lambda Fix (Stratagene) were plated on host strain Le392 and probed with the 1.1kb DOR-1 Pst/Xba I fragment, which contains primarily the coding region.

The conditions for hybridization were of fairly low stringency: 50% formamide/6 X SSC, overnight at 37°C. The washes were performed also at low stringency: 2 X SSC, 0.1% SDS at room temperature.

One mouse clone and three human genomic clones were isolated and purified by sequential rounds of hybridization and plaque purification. DNA preparation and restriction analysis showed that the three human clones had very different EcoRI digestion patterns. The 1.1kb opiate receptor probe hybridized to a different single EcoRI band in Southern blot analysis for each clone. These results indicated preliminarily that three different genes were represented by the human genomic clones which were designated H3, H14 and H20 (see Figures 8a, 8b, 8c and 8d). Each of these clones was deposited on 13 August 1993 at the American Type Culture Collection, Rockville, Maryland, under conditions of the Budapest Treaty. All restrictions on access to these deposits will be irrevocably removed at the time a patent issues in the United States on the basis of this application. The ATCC deposit numbers are _____ for H3, _____ for H14, and _____ for H20.

The H3, H14 and H20 clones were digested into smaller fragments by EcoRI and TaqI and then shotgun cloned into the appropriate site of Bluescript for sequencing. The partial nucleotide sequence for H3 is shown in Figure 8a; the partial nucleotide sequence of H14 is shown in Figure 8b; the partial nucleotide sequence of H20 is shown in Figure 8c.

The three genomic clones were mapped by *in situ* hybridization on human metaphase chromosomes by Dr. Glenn

Evans of the Salk Institute. H3 maps to chromosome 1P; H14 maps near the centromere of chromosome 8, and H20 maps to chromosome 6. Comparison of sequence data obtained as described above with the published sequences for the murine counterparts referenced hereinabove, and with the DOR-2 clone described hereinbelow, confirmed that: (a) H3 encodes the human delta opioid receptor; (b) H14 encodes the human kappa opioid receptor and (c) H20 encodes the human mu receptor. In addition, H20 appears to contain a CACACA marker (Figure 8d) which provides a means to track the inheritance of this gene.

The genomic clones were digested into smaller fragments by EcoRI and TaqI, then shotgun cloned into the appropriate site of Bluescript for sequencing.

Example 7

Isolation of Opioid Receptor Clones From Additional Organisms

In order to isolate the opioid receptor from mammalian brain cells, for example human brain cells, a random-primed human brainstem cDNA library in λ Zap (Stratagene) was screened using the murine cDNA encoding the DOR-1 described herein. Positive plaques were purified and rescreened. Individual positive clones are sequenced and characterized as above.

Example 8

Determination of Probable Antigenic Sequences

By evaluating the amino acid sequence of the opioid receptor encoded by DOR-1 with the MacVector (I.B.I.) antigenic index, and the antigenic index in

accordance to Jameson, B. and H. Wolf, Comput Applic in Biosci (1988) 4:181-186, the following underlined sequences of the delta opioid receptor were determined to have a high antigenic potential:

NH_2MELVPSARAELQSSPLVNLSDAFPSAFPSAGANASGSPGARSASS
LALAIAITALYSAVCAVGLLGNVLVMFGIVRYTKLKTATNIYIFNLAL
ADALATSTLPFQSAKYLMETWPFGELLCKAVLSIDYYNMFTSIFTLTM
MSVDRYIAVCHPVKALDFRTPAKAKLINICIWVLASGVGVPIMVMAVT
QPRDGAVVCMLQFPSPSWYWDTVTKICVFLFAFVVPILIITVCYGLML
LRLRSVRLLSGSKEKDRSLRRITRMVLVVVGAFVVCWAPIHIFVIVWT
LVDINRRDPLVVAALHLCIALGYANSSLNPVLYAFLDENFKRCFRQLC
RTPCGRQEPGSLRRPRQATTRERVTACTPSDGPGGGAAA-COOH.

The N-terminal sequence is extracellular, the other four sequences are predicted to be intracellular.

Example 9

Recovery of the Murine Clone DOR-2 (mMOR-1)

A cDNA library prepared from mouse brain in λgt10 was probed using the low-stringency conditions of Example 6 using DOR-1 as a probe. One clone was recovered, inserted into Bluescript and sequenced. Northern and Southern blots indicated divergence from DOR-1. This clone, designated DOR-2, represented a new gene. DOR-2 hybridized to a different pattern of neurons than did DOR-1 and showed greater labeling of the striatum. Expression of DOR-1 by insertion into the vector pCDNA and transfection into mammalian cells produced cells which bind morphine, indicative of a mu-receptor. The cells also bind the nonselective opiate antagonist diprenorphine. The identity of DOR-2 (mMOR-1) as that of a mu receptor was confirmed by the displacement

of ^{3}H-DPN by nanomolar concentrations of the mu-selective ligands morphiceptin, DAMGO and morphine. The delta selective ligands DPDPE and deltorphan did not displace the binding and naloxone had the expected high affinity. The partial sequence designated H20, described in Example 6, was substantially similar to DOR-2. The partial sequence of DOR-2 is shown in Figure 9.

Figure 10 shows a comparison of the amino acid sequences of murine delta receptin with the rat mu and kappa receptors. There are extensive regions of homology.

Example 10

Isolation of ORL-1

A human brain stem cDNA library was obtained from Stratagene and probed using low-stringency hybridization with the murine DOR-1 sequence shown in Figure 5, under stringency conditions of 50% formamide at 37°C with washes of 1 X SSC at 37°C. A partial cDNA clone encoding ORL-1 was obtained and completed at the 5' end by RACE using cDNA obtained from human brain. The DNA sequence obtained for ORL-1 is shown in Figure 11 and is identical to that reported by Mollereau *et al.* (*supra*). ORL-1 has approximately 44% amino acid identity to the mu receptor.

In addition to ORL-1, three clones for ORL-2 were obtained and a full-length clone was assembled from two overlapping clones. The sequence of one of the ORL-2 clones was identical to that reported by O'Dowd *et al.* (*supra*) while the other had a base change at Leu^{129} which did not result in an alteration of amino acid sequence.

Figure 12 compares the protein sequences of three cloned opioid receptors and ORL-1 and ORL-2.

Multiple PKC and PKA cites in the third intracellular loop of ORL-1 are similar to those in the delta opioid receptor. However, a His residue present in the sixth transmembrane domain of all the opioid receptors is absent in ORL-1; this His residue may play a role in aromatic interaction with ligands and may be critical for opioid receptor binding.

Mollereau *et al.* (*supra*) have shown that a stable cell line transfected with ORL-1 shows etorphine-induced cyclase inhibition. This inhibition is reversible with diprenorphine, although labeled diprenorphine binding to ORL-1 has not been shown. In addition, ORL-1 has two Asn-linked glycosylation sites in the N-terminal extracellular domain as shown in Figure 12.